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14. ABSTRACT
Surgery of the spine to fuse the vertebral bones is one of the most commonly performed operations with an estimated 350,000 Americans undergoing this surgery annually with estimated costs of $60 billion. Current procedures are highly invasive with limited success. The goal of this study is to develop a safe efficacious system for inducing spine fusion which will eliminate the need for invasive surgery. We have currently developed a cell based gene therapy system that can induce rapid bone formation at a targeted location which is independent of immune status of the model. This system relies on adenovirus transduced cells expressing bone morphogenetic protein 2 to induce bone formation leading to vertebral fusion after delivery into the paraspinous musculature. To prolong cell survival and insure cells are maintained at the target site, we have encapsulated them in a non-degradable hydrogel material. This provides additional safety by eliminating direct injection of the virus through cell delivery, and prevention of cell diffusion, through encapsulation. Here we provide preliminary data; demonstrating spine fusion using this system at 6 weeks after induction. This is the first step in demonstrating efficacy, a critical component of preclinical testing. Thus with validation of our hypothesis, this approach can now be developed as a safe and efficacious gene therapy system for spine fusion, thus circumventing the need for costly invasive surgery.

15. SUBJECT TERMS
BMP2, Spine fusion, Hydrogel, Gene Therapy, Adenovirus

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Introduction: Surgery of the spine to fuse the vertebral bones is one of the most commonly performed operations with some 400,000 Americans undergoing this type of surgery annually in the United States. The estimated cost associated with such procedures exceeding $60 billion annually demonstrating this to be a significant problem. In the most common form, posterolateral fusion, the paraspinous musculature is stripped and the bone decorticated, resulting in significant pain, reduced stability afforded by these muscles, and disruption of the blood supply to both bone and muscle. Further, success rates for fusion range from 50-70% depending on how many levels are fused and the number and types of attendant complications. We recently demonstrated that transduced cells expressing high levels of bone morphogenetic protein 2 (BMP2) in skeletal muscle could rapidly recruit and expand endogenous cell populations to initiate all stages of endochondral bone formation, with mineralized bone forming within one week of implantation. The central hypothesis of this application is that posterolateral spine fusion can be successfully achieved with only minimally invasive percutaneous techniques and without a scaffold, by collecting cells from patient's, transducing them to express BMP2, encapsulating the cells with hydrogel material, and then delivering them to the fusion site. If added structural stability is required, the injectable hydrogel will be crosslinked *in vivo* with a small fiber-optic light source. Successful completion of this project would advance the current state of gene therapy in this field by eliminating the search for an optimal osteoprogenitor cell and scaffolding.

Body: The central hypothesis of this application is that posterolateral spine fusion can be successfully achieved with a novel and simple minimally invasive percutaneous technique. We propose that this can be done by transducing human fibroblasts to express an osteoinductive factor (bone morphogenetic protein 2 or BMP2), encapsulating the cells with hydrogel material, and then delivering them to the fusion site. This injectable material will be a liquid, but once in place can be crosslinked with a small fiber-optic light source. We have developed three specific tasks to accomplish our goals.

Task 1: To produce high levels of BMP2 from human mesenchymal stem cells transduced Ad5F35BMP2 adenovirus in the presence of tetracycline carrying a red luciferase reporter gene.
Viability and expression of the BMP2 will be monitored using an Ad5F35BMP2-IRESCBRluc vector that can be readily tracked using a CCD camera system, and will allow us to follow transgene expression within live animals. BMP2 expression will be regulated by tetracycline, to confirm optimal timing for bone induction. Experiments will also be performed to identify the cells responding to BMP2 and determine if this process is local or systemic. This will be accomplished using SMAD 5 "biosensor" mice in which response to BMP2 specifically leads to fluorescence emission in those cells.

a. To determine if sustained expression of BMP2 is more efficient at inducing rapid bone formation than a pulse of expression using the tetracycline regulated vectors. **(Months 0-12)**

We initiated studies to look at regulated expression of the BMP2 in the animal. We had trouble with our adenovirus vector that possessed the tetracycline regulatory elements. Thus we had to reconfigure the clone, as well as simultaneously moved towards implementing a tamoxifen regulated vector in which the tamoxifen regulated promoter drives BMP2. We estimate both of these projects which have been underway for several months, to be completed this year.

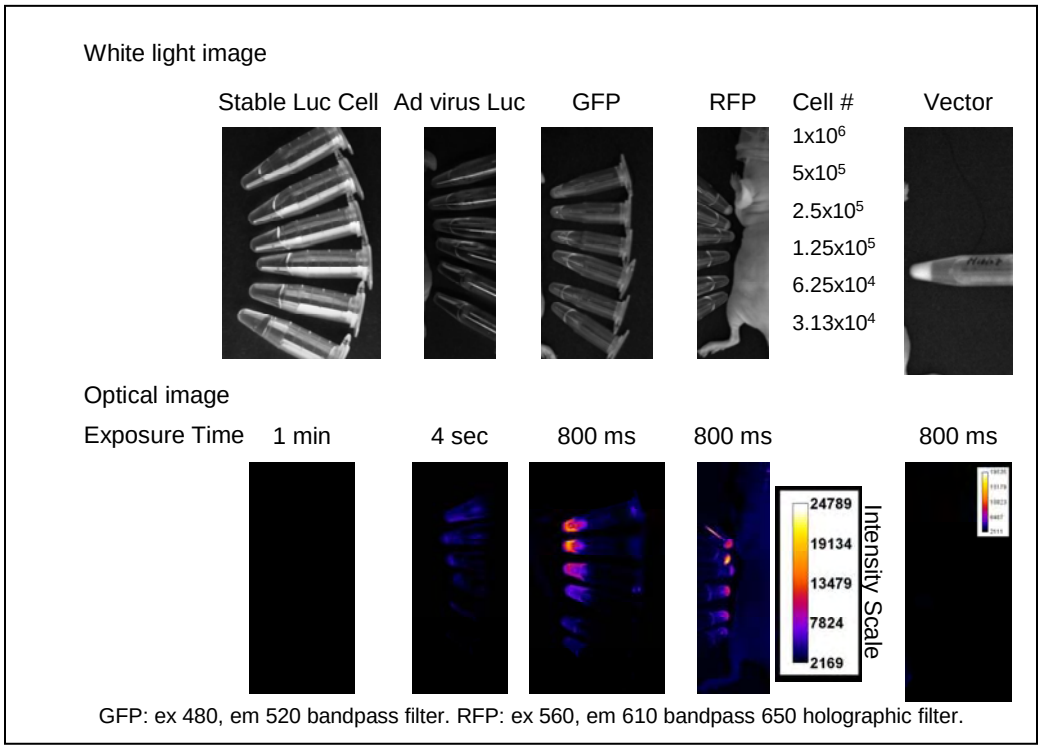
b. To determine if longer expression times of BMP2 from cells embedded in hydrogel and longer cellular viability lead to more rapid bone formation than the rapid but short burst of BMP2 release obtained from the cells directly injected. **(Months 9-12)**

We initiated studies to look at regulated expression of the BMP2 in the animal. We had trouble with our adenovirus vector that possessed the tetracycline regulatory elements. Thus we had to reconfigure the clone, as well as simultaneously moved towards implementing a tamoxifen regulated vector in which the tamoxifen regulated promoter drives BMP2. We estimate both of these projects which have been underway for several months, to be completed this year.

c. To demonstrate the termination of BMP2 expression using an Ad5F35tet-BMP2-IRESCBRLuc vector in which expression can be tracked through live animal imaging. **(Months 12-24)**

a. The BMP2 expression can be tracked by expression of click beetle luciferase and a CCD camera. The vector (Ad5F35BMP2-IRESCBRLuc) is constructed such that the CMV promoter drives transcription of both the BMP2 gene but also an internal ribosomal binding site and then the red luciferase transgene so that both proteins are translated from the same transcript. Thus detection of the luciferase confirms production of the BMP2 transcript at that location.

We are working on developing the *in vivo* tracking system to efficiently follow the BMP2 transduced cells, and verify their location within the hydrogel materials. We are also currently developing a near infrared dye IR800 that will enter cells and bind to a peptide moiety known as halo tag (Promega Corp). We believe the near infrared will provide the greatest sensitivity. However, we are including preliminary data comparing three commonly used cell tracking modalities. In figure 1A, we injected varying numbers of cells transduced with an adenovirus (5000 vp/cell) possessing the DSRed, GFP, or click beetle red luciferase (CBRLuc) transgenes. As can be seen in figure 1, very little luciferase could be detected from the highest numbers of cells, and (fig 1B) none of the concentrations of cells were detected in the mice itself. Alternatively all concentrations of cells could readily be detected in the DSRed samples, and cell numbers ranging from 1 x 10^6 to 6 x 10^4 cells being detected in the animal after subcutaneous injection. We propose that introduction of the near infrared dye will allow us to detect as few as 100 cells. We propose to complete these experiments in the next quarter, and will then initiate the tracking experiments to determine both cell viability and exact location of the injected materials.



GFP: ex 480, em 520 bandpass filter. RFP: ex 560, em 610 bandpass 650 holographic filter.

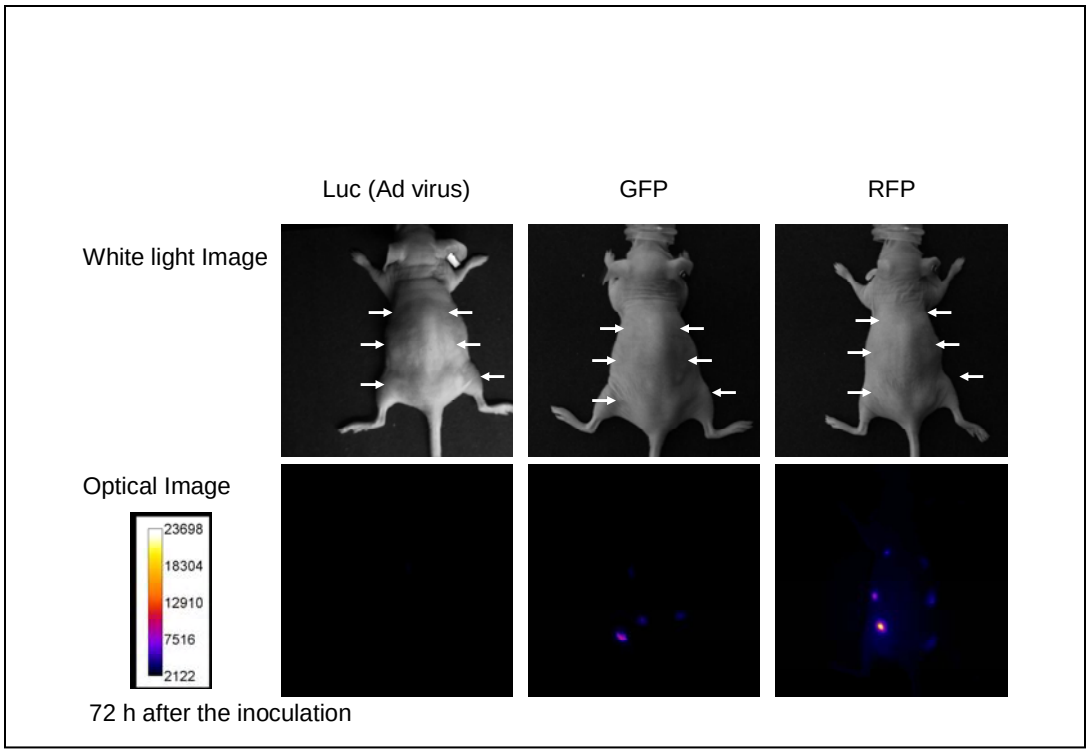


Figure 1A: Varying number of cells transduced with Ad5CBRLuc, -GFP, -DSRed, and Control "empty cassette vector" **B**: Same cells after subcutaneous injection into the mice.

We have currently constructed the Ad5E1BMP2 E3dsRED vector for initial testing. We have found that this reporter to be the most sensitive, and easiest for use in the hydrogel material. We have thus initiated the experiments to track the injected cells and compare the temporal and spatial expression of dsRed *in vivo*, to that obtained from cells in the hydrogel material as described in Aim 1. One anticipated problem was the ability to see the material through the muscle tissues. Further the hydrogel may in fact shield the dsRED signal to some extent. Therefore in the initial experiment we went ahead and started with a single cell number of 5 x 10^6 cells either encapsulated in hydrogel, or directly injected into the muscles. In these experiments human MRC-5 cells were transduced with the Ad5E1BMP2E3dsRED and then either encapsulated in hydrogel, injected and photopolymerized in place, or directly injected into the hind quadriceps muscle. Due to difficulties with the animal facilities, we did not follow the same mice throughout but rather had different mice per time point. However, we are currently set up to do a second study in which we will follow the same animal for a longer period of time.

As can be seen in figure 1, the DSred expression in the cells was readily detectable in all animals. In all cases we used both hind limbs, however, as can been seen in figure 1, often the signal could often only be detected in either the dorsal or ventral view rather than both. As is the case in animal 1, both legs have robust signal only in the two different views, whereas in animal 2 the signal is only detected in the ventral view in both legs. Interestingly, we did not see much back ground signal from the hydrogel material, in fact the signal seemed to be somewhat shielded as expected, however, still detectable in each limb (as marked by the arrows).

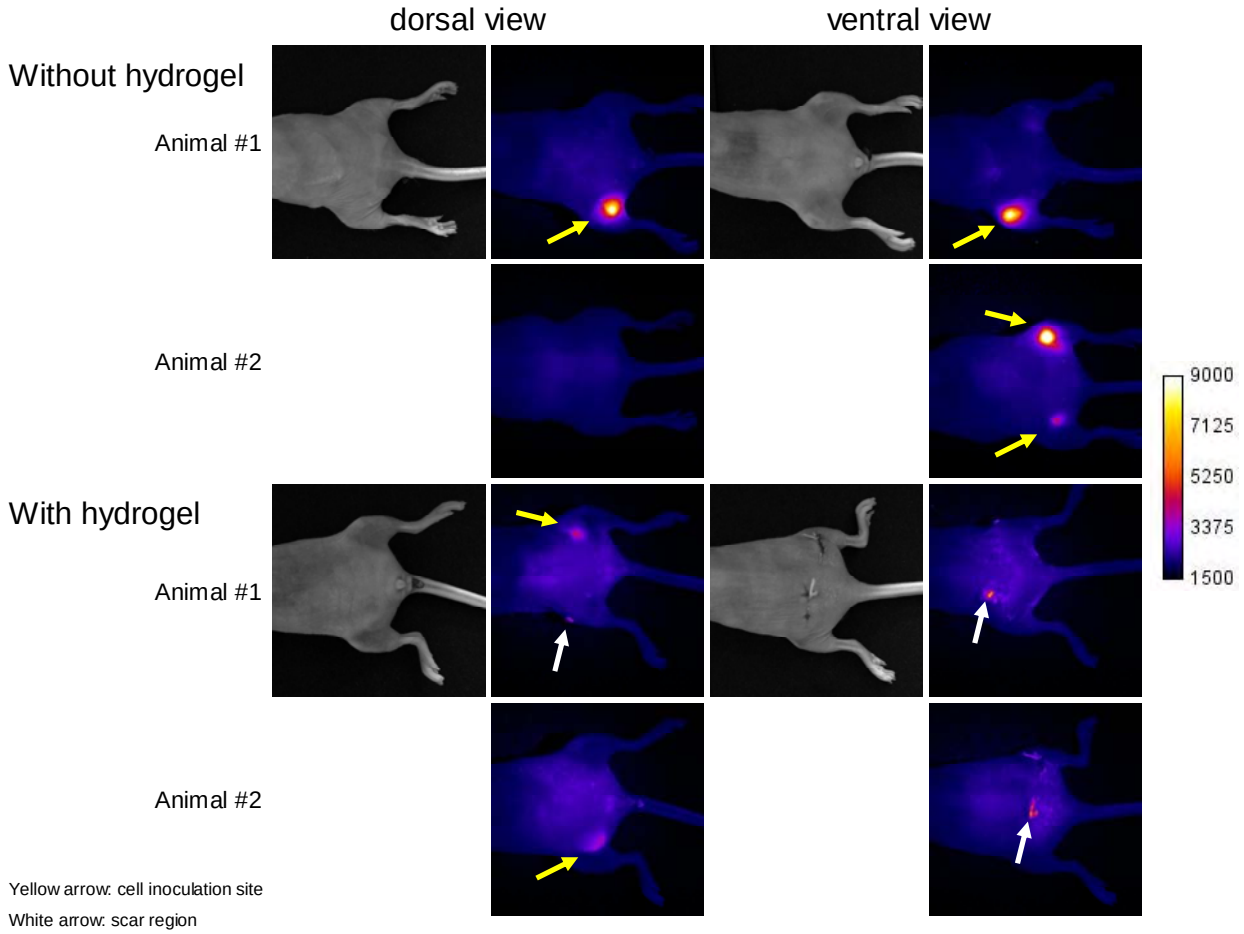


Figure 2: Live animal optical imaging of nude mice which received 5 x 10^6 MRC5 cells transduced with Ad5BMP2 and encapsulated or unencapsulated in hydrogel. In these experiments the cells were directly injected into the quardriceps muscle or fibers were separted, the hydrogel and cells injected, and then the fiberoptic light shown on the gel material to crosslink the material in place. The mice were then imaged 4 days later.

d. To determine what the responder cells to the BMP2 are proximal or more distal to the hydrogel encapsulated delivery cells by performing live animal confocal imaging which follows the translocation of a Smad 5 to the nucleus. **(Months 24-48)**

We initiated these experiments earlier than proposed since the set up of imaging this *in vivo* model is a large undertaking and will require a significant amount of time to validate. Initial experiments have been done to compare and pinpoint the changes in phosphoSmad signaling in tissues receiving the Ad5BMP2 transduced cells, as compared to those receiving cells transduced with control virus. This analysis was done in tissue sections to provide preliminary data before setting up the in vivo imaging. These preliminary experiments will allow us to not only pinpoint locations within the tissues to focus, but also provide us with a time frame of signaling, thus providing incite for the in vivo studies. Figure 3, shows immunohistochemical staining for phosphoSmad using an antibody specific to the active phosphorylated form in tissue sections taken from tissues isolated four days after injection of the transduced cells. These initial experiments were done in wild type (C57BL/6) mice. The phoshpoSmad mice are currently constructed and are being breed to expand there numbers, as well as further characterized as to their exact phenotype.

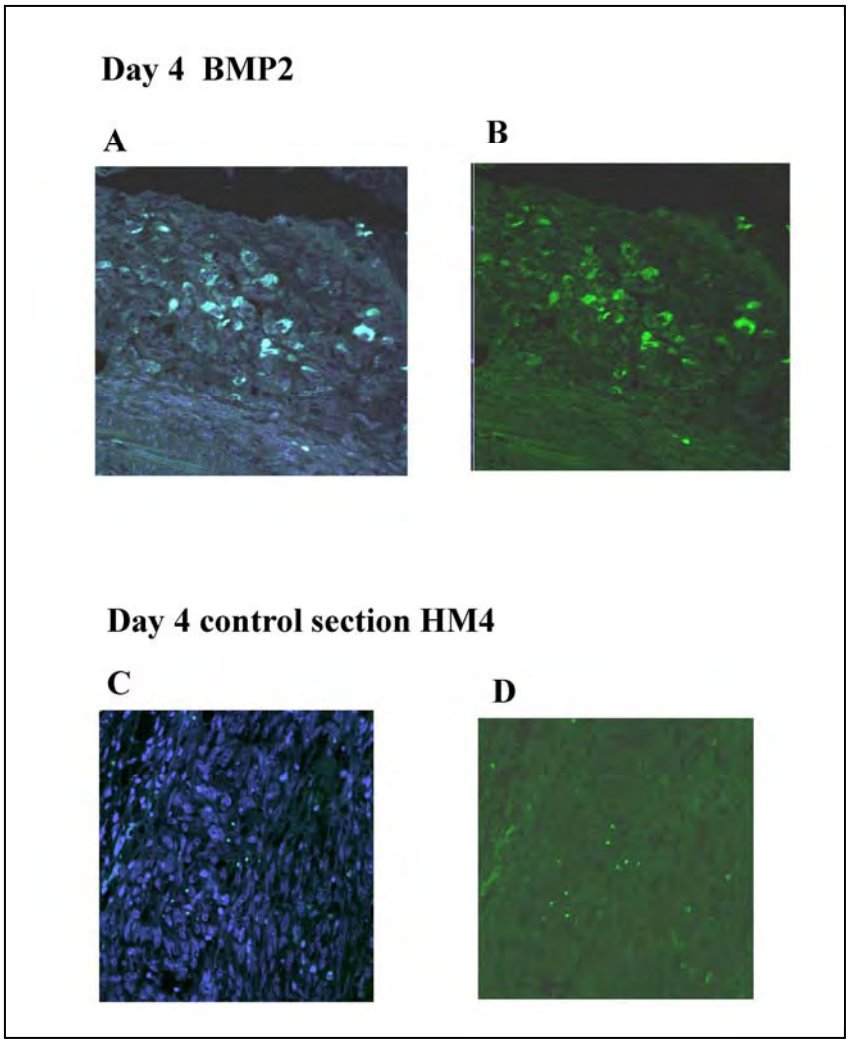


Figure 3: Immnuno-fluorescence staining on tissues isolated four days after receiving Ad5F35BMP2 transduced cells (A and B) or cells transduced with a control virus Ad4F35HM4 (C and D). Sections B and D are stained with Phospho-Smad1/5/8 antibody (1/100 dilution, Cell Signaling technology) and a secondary antibody anti-rabbit Alexa Fluor 488 conjugated. Sections A and C and stained with Phospho-Smad1/5/8 and counterstained with DAPI

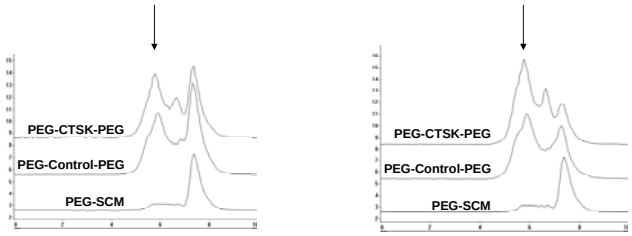
Task 2: To design an optimal hydrogel material that will rapidly promote endochondral bone formation and be capable of removal through bone remodeling processes. In addition to BMP2 transduced cells, we propose to include peptides essential to the recruitment and migration of osteoprogenitors for bone and cartilage. Selective protease sites will also be introduced into the hydrogel to allow for osteoclast selective degradation during bone remodeling. We propose to do this by incorporation of calcium into the material and inclusion of cathepsin K protease cleavage sites into the material. Inclusion of these factors in the hydrogel will provide a mechanism for removal of the hydrogel once bone has formed by using the normal bone remodeling process.

a. Optimize and develop a hydrogel that can be specifically degraded by osteoclasts. **(Months 0-24)**
We have synthesized the peptide MGPSGPRG using a 431A solid-phase peptide synthesizer (Applied Biosystems, Foster City, CA), but have not yet incorporated the peptide into the hydrogel. In order to make degradable PEG, we start with PEG-DA-SMC (succinimidyl carbonate). The PEG-SMC is conjugated with our peptide in order to get PEG-PEPTIDE-PEG. So, we expect to obtain three peaks representing the completely conjugated product: PEG-PEPIDE-PEG, incompletely conjugated product: PEG-PEPTIDE and unconjugated product: PEG-SMC (Figure 4).

Figure 4: Results of HPLC analysis of the conjugation of peptide with the

Synthesis of Cathepsin K degradable PEGDA hydrogel

- Cathepsin K-sensitive sequence (CTSK):
  - MGPSGPRGK
- Control sequence:
  - MPGSPGGRK
- Conjugate with 3400 Da PEG-SCM



Dialyzed overnight **Dialyzed for four days**

We have run GPC tests on it and the results indicate that there is still unconjugated PEG, ie: we have PEG-Peptide or free PEG in the preparations. We have tried changing the ratio of peptide to PEG, changing the length of conjugation time, increasing the pore size of the dialysis membrane and changing the length we dialyze the conjugation products. These have led to a higher concentration of PEG-PEPTIDE-PEG as indicated by the GPC results (Figure 4). We have cathepsin K (Calbiochem; Cathepsin K, His•Tag®, Human, Recombinant, *E. coli*) and are ready to test their degradability.

The next step in developing this material was to test whether the conjugation of the cathepsin K site is enough to degrade a polymerized peptide. We placed microbeads of the hydrogel in the 25 µL cathepsin K vial (stock solution 0.2 mg/mL cathepsin K) incubated at 37°C. The PEG-peptide-PEG hydrogels (where "peptide" refers to the cathepsin K degradable peptide) were tested for swelling and degradation in PBS and cathepsin K, respectively (n=1). A swelling hydrogel typically has a logarithmic curve, like the PBS curve, where the weight of the gel increases and reaches a maximum. A degrading hydrogel will initially show an increase in weight as broken bonds allow more water in, allowing the hydrogel to swell more. Then the swollen weight will drop dramatically. Figure 5A and B shows evidence that the hydrogels are at least partly degradable. However, the results also suggest that the material cannot be completely degraded suggesting the PEG-Peptide-PEG is limiting, or that the sites are not efficiently digested. To circumvent the latter, we are

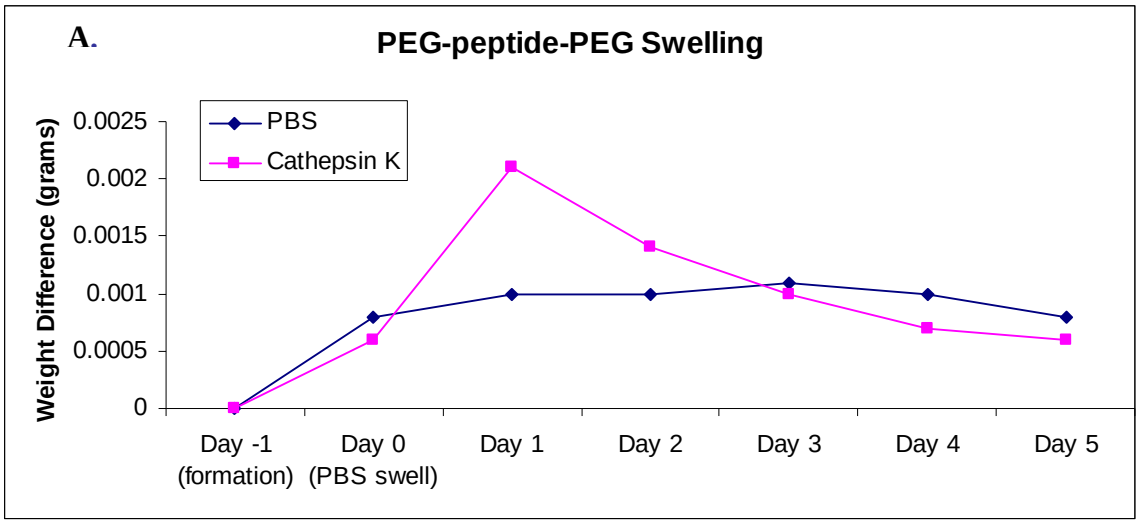
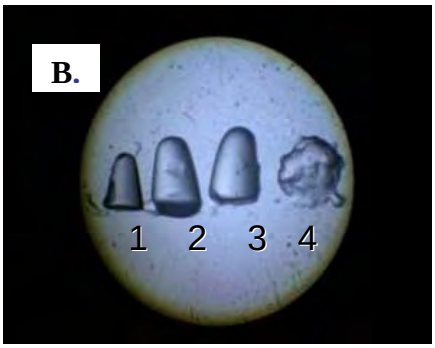


Figure 5: Swelling test of the Cathepsin K degradable peptide. **A.** Weight changes upon swelling during degradation. **B.**Hydrogels after swelling and degradation. (1) PEG-CTSK-PEG in buffer w/o proteinase K; (2) PEG-CTSK-PEG w/ 0.125 mg/ml; (3) PEG-control-PEG w/ 0.125 mg/ml; (4) PEG-CTSK-PEG w/ 0.250 mg/ml after compression.



resynthesizing the peptide to include additional 3 glycinces on either end to lengthen the protein region and ensure that cathepsin K can reach it efficiently within its PEG backbone. Hopefully with the additional amino acids on the peptide site, any constraints the protease may have in binding for digestion of the site will be alleviated.

We have been recently testing the new protease site which now has a glycine repeat sequence was designed into the peptide in order to act as a spacer. Thus the modified peptide sequence is GGGMGPSGPWGGK, see figure 6. The W is tryptophan that we will use to track the degradation of the hydrogel by measuring tryptophan release into the media by taking Ultra-Violet/Visible (UV/VIS) Spectrophotometry absorbance measurements at 280 nm.

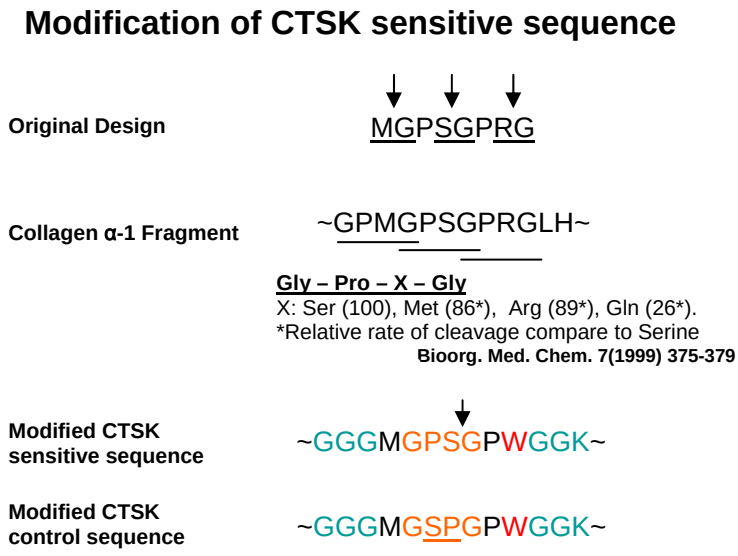


Figure 6: Schematic of the changes made in the peptide to introduce the cathepsin K protease site into the PEG-DA hydrogel.

Rather than incorporate this new peptide immediately into a PEG backbone, it was tested directly. We ran mass spectrometry on the peptide in order to see if the predicted molecular weight (1143.43) of the peptide was present. We found a peak at 1143.9, indicating success of the peptide synthesis. Next, we incorporated the peptide with activated cathepsin K and repeated the mass spectrometry. The 1143.9 peak was now absent, and present were predicted cleavage products (figure 7)

CTSK intact and degraded

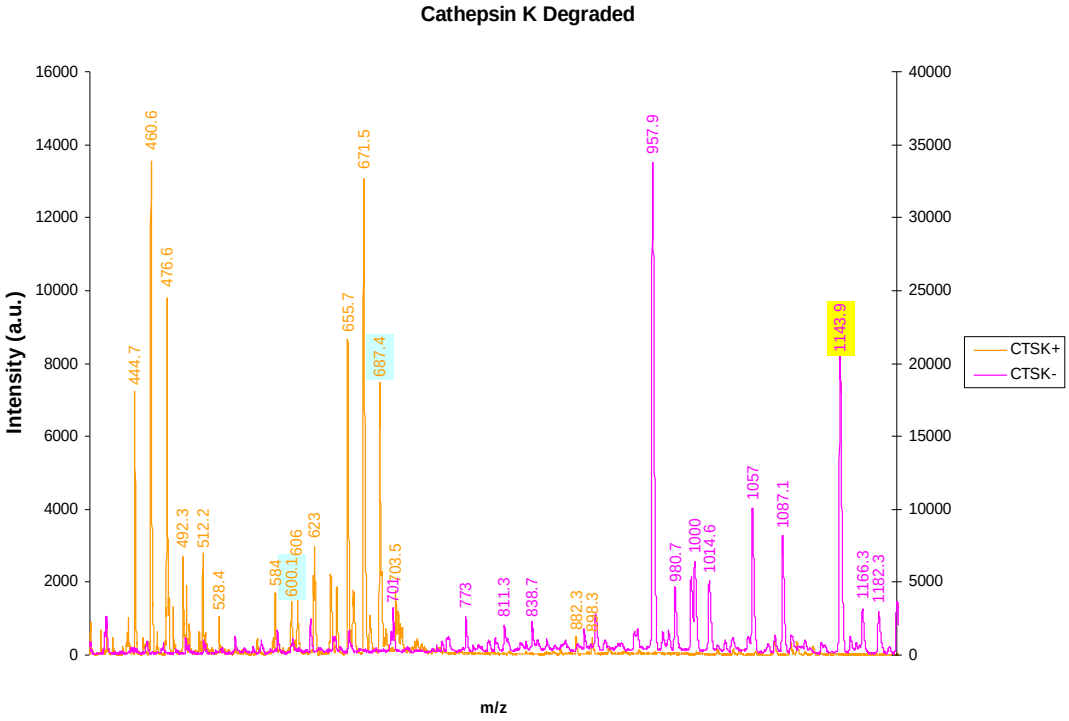


Figure 7: Mass spectrometry of the peptide consisting of the cathespin K protease site (CTSK) before and after cleavage with the protease.

We also discovered that our control peptide had a cleavage site that we did not immediately notice until after it had been made. We will need to re-prepare the control to eliminate this site.

We proceeded with degradation tests when prepared hydrogels degraded almost immediately in proteinase K. To prevent artifact from increased enzyme concentration due to evaporation over time, we used a concentration of proteinase K that would degrade over hours, rather than days, and matched that concentration for the cathepsin K degradation tests. We were pleased to see that the tryptophan release test suggested that the hydrogels would be completely degraded by the cathepsinK similarly to when protease K was added (figure 8). This demonstrates that they had adequate incorporation of the peptide into the hyrodgel and that the selective enzyme cathespin K could cleave it.

More studies are needed to demonstrate the selective cleavage using enzymes that do not cleave. These studies have already been initiated. Further, we will also run

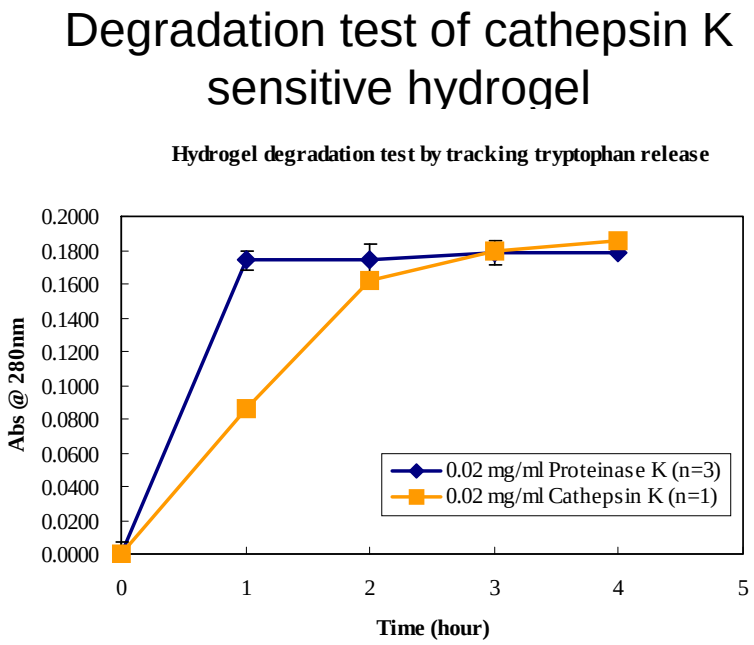


Figure 8: Trytophan release demonstrated complete degradation of the hydrogels by either the proteinase K or cathepsin K.

concentration curves to determine the minimum amount of cathepsin K required for efficient degradation. Once determined, we will run wet weight comparison experiments. First the hydrogels will be weighed and then allowed to swell in HBS with 1 mM $CaCl_2$ and 0.2 mg/mL sodium azide at 37°C for 24 h. Each hydrogel sample will then incubated at 37°C with HBS and the minimum concentration of cathepsin K required, 0.2 mg/mL proteinase K, 0.2 mg/mL plasmin, or no protease solution. Proteinase K will be used as a positive control for nonspecific proteolytic activity; plasmin will be used to determine selectivity of specific proteases for the cathepsin K site (negative control) and HBS without protease to demonstrate stability of the conjugated hydrogel. Degradation will then be evaluated by monitoring changes in the wet weight of hydrogels over time. The enzyme solution will be changed at 24 h intervals.

We are also setting up the RAW264.7 monocyte cell line that can be differentiated into osteoclasts in the presence of RANKL. We will incubate the hydrogels in the presence of the osteoclasts, as well as control fibroblasts including an osteoblast cell line. We will also seed some of the gels with cells expressing the DSRed BMP2 construct, and measure cell viability, DSRed expression and BMP2 secretion during the degradation by osteoclasts. With completion of these experiments we propose to publish this early development and optimization work, and then head to *in vivo* experiments within the animals.

b. Test these gels *in vivo.* **(Months 36-48)**
c. Engineer cellular binding sights within the hydrogel to determine if this improves, cell viability of the transduced cells, and in turn BMP2 expression, and to tentatively enhance the migration of mesenchmyal stem cells to the sight of bone formation. **(Months 24-36)**
d. Engineer proteins that may enhance the BMP2 bone inductive response, such as VEGF-A or -D and compare with gels without additional proteins. **(Months 36-48)**

Task 3: To achieve posterolateral spine fusion by percutaneous injection of the encapsulated Ad5F35BMP2 transduced senchymal stem cells into the paraspinous musculature of both rats and mice. Spine fusion will be assessed in both a rat and mouse model by both histological and radiological analyses over time and confirmed by both microCT and biomechanical testing. The results will also be compared to a parallel murine system which is immunocompetent. These experiments will provide essential preclinical data in two different animal models.

a. Obtain approvals through the DOD institutional review board for approval to work with the human mesenchymal stem cells. **(Months 0-12)**

This was completed within the first few months of the award.

b. Once approved we will start to utilize these cells in all hydrogel formulation experiments as described in Task 2. **(Months 12-36)**

The clinical use of BMPs in human spinal fusion surgery has been largely limited to the surgical implantation of BMPs via traditional open surgical techniques that involve extensive soft tissue dissection and local decortication of the bone. Frequently these procedures are accompanied by surgical bone graft harvest from the pelvis. The creation of a bony fusion by means of the percutaneous injection of biologically active material, without extensive surgical dissection and bony decortication, would have many clear clinical advantages. This could potentially markedly decrease the pain and recovery time for patients undergoing these procedures. This approach could also drastically reduce blood loss, complication rates, and cost.

Our initial testing demonstrated the ability of the heterotopic bone formation induced by our model, to fuse to at least one vertebral bone, without prior decortification or injury. Since our goal is to develop the cell based gene therapy system in combination with PEG-DA hydrogel for percutaneous delivery resulting in optimal spine fusion, we set up a series of animals to evaluate over time as to the degree of bone formation, as well as resultant fusion. Briefly, MRC5 cells (fetal lung cells, ATCC) were transduced with (2500vp/cell) of Ad5F35BMP2 or Ad5F35control and then injected or preformed hydrogel beads implanted into the hind limb quadriceps muscle of NOD-SCID mice (n=8/group). Animals were euthanized 6 weeks after injection of the transduced cells and resultant bone formation analyzed by microCT and histology.

PEG-DA (10,000 Da) was synthesized by a reaction of PEG with acrylol chloride as previously described[3]. The dried polymers were then dissolved in water and filter sterilized prior to use. Hyrdogel beads were photopolymerized by combining 0.1 g/ml PEG-DA (10% w/v) with 1.5% (v/v) triethanolamine/HEPES buffered saline (HBS, pH 7.4), 37 mM 1-vinyl-2-pyrrolidinone, 10 mM eosin Y and MRC-5 cells transduced with

Ad5F35BMP2 or Ad5F35Control virus for a final concentration of 10 millions cells/150 uls. The cell-seeded gels were crosslinked by exposure to visible light for 2 mins.

Heterotopic bone formation was observed in all groups receiving cells transduced to express BMP2. However, analysis of the spines suggested that placement of the biomaterials was critical to obtaining rapid fusion. As can be seen in table 1, 6 of 7 samples receiving the hydrogel encapsulated cells expressing BMP2 appeared to fuse to at least one vertebral bone, whereas in no case did we observe any bone formation or fusion in the control animals receiving either hydrogel, or hydrogel encapsulated cells transduced with control virus.

	Hydrogel encapsulated Ad5F35BMP2 transduced cells	Hydrogel encapsulated Ad5F35control transduced cells	Hyrdrogel alone	Ad5F35BMP 2 transduced cells	Ad5F35Control trasnduced cells
Animals/group	**8(7) ***	8	8	8	8
Number with one or more tentative fusions to adjacent lumbar vertebra	**6**	0	0	4	0
Percentage of vertebral fusion	**86%**	0%	0%	50%	0%

* One animal in this group died shortly after implantation

In comparison, microCT analyzed of tissues receiving Ad5F35BMP2 transduced cells directly, resulted in the heterotopic bone fusing to one or more lumbar vertebra in 4 of 8 samples. Interestingly, this group had fewer tentative fusions; but 3 of the 4 positive tissues, formed bony fusion with solid bony bridging spanning adjacent vertebrae accomplishing the goal of spinal fusion. Figure 9, shows representative images from each group.

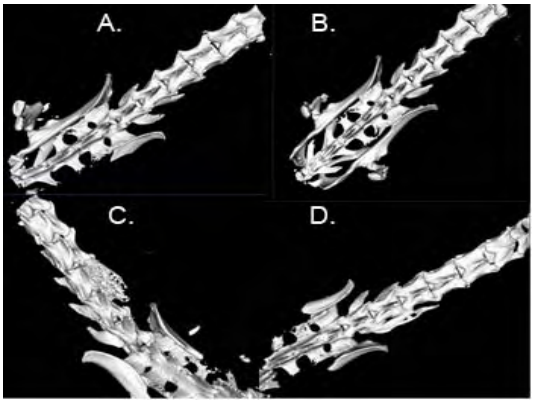


Figure 9: MicroCT analysis of spines isolated 6 weeks after implantation of hydrogel encapsulated cells transduced with **(A)** Ad5F35Control, **(C)** **A**d5F35BMP2, or direct injection of cells tranduced with **(B)** Ad5F35Control or **(D)**Ad5F35BMP2.

The results described here demonstrate that the use of BMP producing cells encapsulated within a hydrogel, readily induce heterotopic bone formation. This heterotopic bone can directly fuse with vertebral bodies without prior surgical exposure or decortication of the bone. Indeed, in these preliminary experiments, some animals developed true bony fusion between adjacent vertebrae. This approach has significant potential for eventual human application.

c. Once the gels have been modified to offer optimal properties for bone formation and removal, we will test these in a murine model of spine fusion. Since we have substantial knowledge of the mouse model, we will initially start to collect data with this system. We will demonstrate the ability to induce spine fusion in the presence of tetracycline. **(Months 24-40)**

d. Analyze the modified injectable hydrogel for optimal volume, in vivo crosslinking, design, selective degradation, and inflammatory reaction using both live animal imaging and histology. **(Months 24-48)**

e. All fusions will be tested both biomechnically as well as radiologically using microCT to confirm the fusion. **(Months 40-48)**

A method to reliably determine if newly mineralized tissue adjacent to the spine has formed a structurally competent bridge between vertebrae is needed to assess new spine fusion technologies in small animal models. Micro-computed tomography can be used to visualize any newly formed mineralized tissue adjacent to the spine, but it can be difficult in some cases to determine if the mineralized tissue is actually integrated with the vertebrae or if it is only overlying the vertebrae. Micro-CT exams also require expensive equipment and

long-scan and post-processing times. To provide a simple and rapid method to directly test whether a fusion was mechanically successful, a mechanical device was developed that creates controlled flexion and extension in a mouse spine, so that spine fusions could be assessed using the same computer-assisted methods that are now widely used to assess spine fusions in human patients. This device was validated in a mouse spine fusion model. Utilization of the device involves embedding the spine and surrounding tissues, after removal from the body, in mold using rapid setting alginate. The embedded spine is then flexed using the device and a micro-radiograph is obtained with a digital Faxitron system. The embedded spine is then placed in extension and a second radiograph is obtained. These two radiographs are then imported into a workstation and analyzed using previously validated computer-assisted technology (Zhao KD, *et al*.2005). The software allows intervertebral motion to be accurately quantified. A successful spine fusion is intended to stop any significant motion between vertebrae. In the human spine, intervertebral motion under 1.5 degrees at any level is considered to be reliable evidence of a solid spine fusion (Hipp JA et al, 2008) using this image processing technology.

To test whether the tentative fusions were actually capable of reducing motion within the spine, we set up some experiments to look at flexion/tension under bending at specific angles. Briefly, three groups of NOD/Scid mice were given an injection of MRC5 cells transduced with 1) Ad5F35BMP2 (2 week analysis), 2) Ad5F35BMP2 (6 weeks), and 3) Ad5F35empty cassette all at 2500 vp/cell. The cells were transplanted into the paraspinous musculature of through direct injection. Group 1 animals were isolated at 2 weeks, while groups 2 and 3 were harvested at 6 weeks after initial induction. The spines were then embedded in an agarose gel material, and placed between two plates for bending analysis (figure 10).

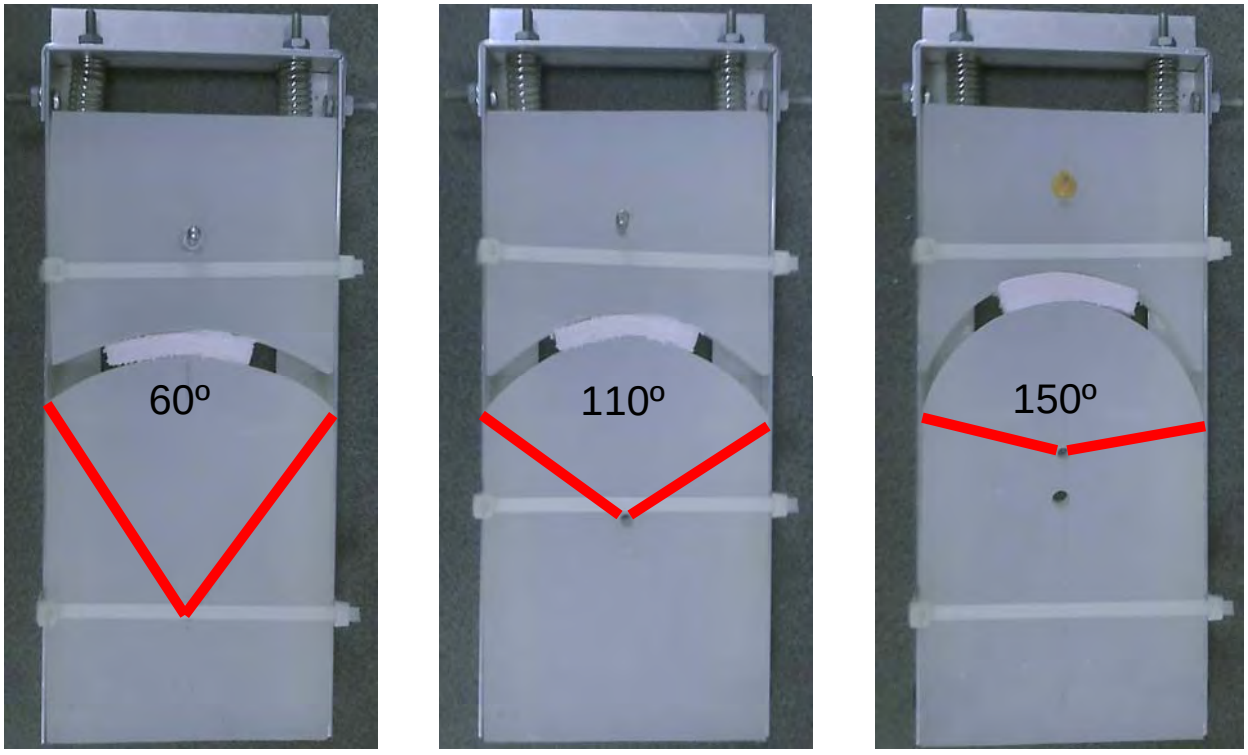


Figure 10. A device for flexing and extension at 60º, 110º, 150º Arcs. The spines were encased in an agarose ge and then flexion/extension was able to be transmitted to encased spine for testing of the fusions.

To determine what to expect for normal spine flexibility in these mice, we subjected a group which had received no treatment to this analysis. As can be seen in figure 11, the values obtained from subjecting the mice to 60°, 110°, and 150° angle varied significantly at the ends of the spine and most likely due to the isolation, and the fact that the spine had been cut. Discs within the center and lower

spine showed significance between 60 and 110 degree shifting with little or no variability, and allowed us to focus on this region for analyzing our fusions.

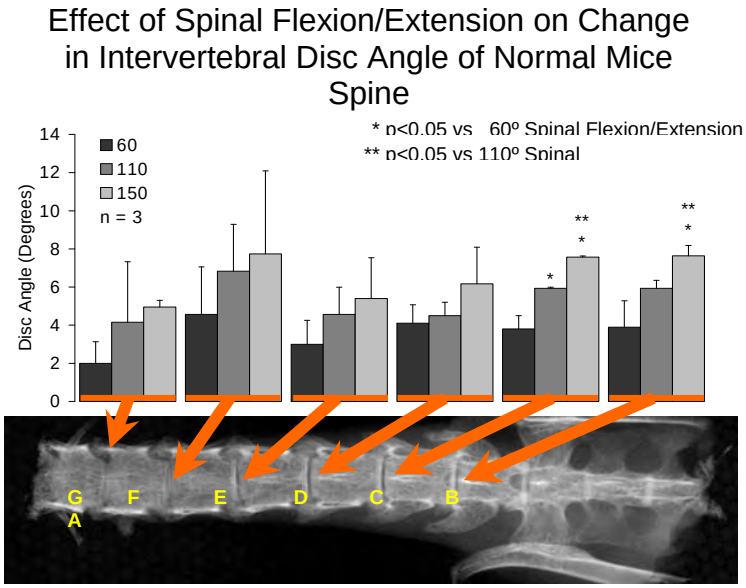


Figure 11: Results of analysis of the disc angle after bending at 60,110, and 150 degree angles. Each column of numbers represents the corresponding disc (arrow). The results suggest more variability in discs on the end of the animal, where the spine has been cut, versus internal or adjacent to the pelvis which remains on these tissues.

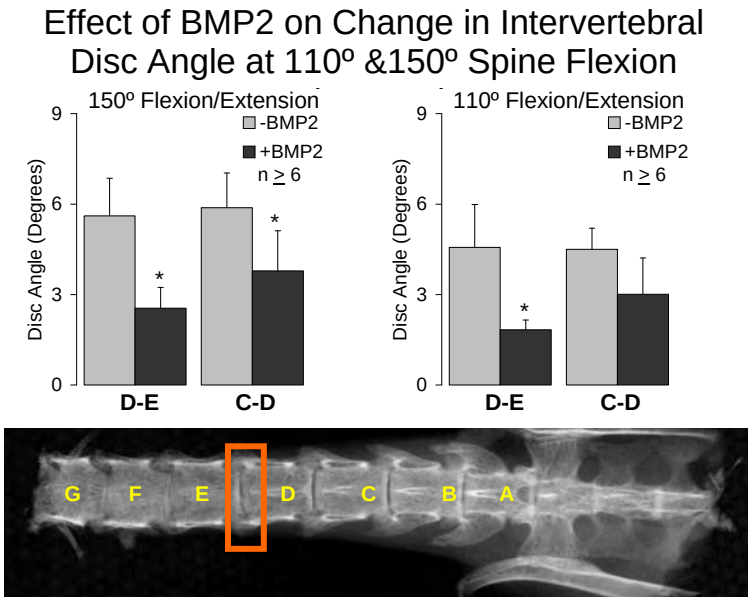


Figure 12: Results of analysis of the disc angle after bending at110, and 150 degree angles in tissues isolated from mice 6 weeks after induction with BMP2. The disc analyzed is listed as the disc between the two lettered vertebra. The disc which provided the most significance in limiting the disc angle is highlighted by the orange box. This sample is representative of the group of fused spines.

Next we analyzed the spines from all groups. In the case of the control, and 2 week BMP2, we did not find any statistical difference from the normal group. Although at 2 weeks we observed heterotopic bone by x-ray and microCT, this bone had not adequately fused to the vertebral bone, and thus did not shown significant differences form the normal spines. However, results from analysis of the group isolated 6 weeks after delivery of the BMP2 expressing cells, showed a significant drop in the disc angle and in turn flexion of the spine. The reduction in flexion is consistent with what has been demonstrated in human spines as a standard for clinical fusion. Figure 12 shows the results of a representative sample from this group.

To verify the amount of intervertebral motion that exists in normal mouse spines using this test method, 9 NOD/SCID mouse spines that had not been exposed to any intervention were tested. Intervertebral motion averaged 5 degrees between each pair of vertebrae in the normal mouse spine. As a gold-standard to determine whether the spines were or were not fused, the mouse spines were immersed in bleach for approximately one hour after the mechanical tests were completed. This process removes all soft tissues from around the vertebral bodies. A nylon string is threaded up the spinal canal midway through this process so that the relative position of vertebrae is maintained. All vertebrae that are not fused together fall-apart during this

process, and all vertebrae that are fused together remain attached (Figure 13). Twenty vertebrae had injections of cells expressing BMP alongside the lumbar spine. Twenty vertebrae had injections of cells expressing BMP alongside the lumbar spine. The spines of 10 mice were analyzed 2 weeks after the injection and the other 10 spines were analyzed 6 weeks after the injection. The average intervertebral motion at those levels confirmed to be fused after the bleaching process was significantly ($P<0.0001$) less than at levels where vertebrae separated after bleaching.

Figure 13: Spine tissue isolated from group 2, which received BMP2 and were isolated 6 weeks after induction. As can be seen the lumbar spine vertebra (five total) were fused in this animal. The sample shown here is representative of the BMP2 group isolated at 6 weeks.

f. Once we have established methodology that leads to reproducible fusion we will further test this in a rat model of spine fusion using athymic rats spine fusion using athymic rats. We will then compare and confirm that these results are similar to those obtained in immuno-competent mice. **(Months 40-48)**

g. To initiate toxicology studies, as potentially outlined in a pre-IND meeting with the FDA. **(Months 24-48)**

Key Research Accomplishments

- We have developed a method for monitoring BMP2 expression in live animals through the use of dsRED. This optical imaging modality appears to be specific and significantly more sensitive than either lucerifase imaging or GFP detection. We have initiated studies to look at the regulated BMP2 carrying a dsRED gene and compare bone formation in the presence of long term versus short term BMP2 expression.
- We have developed a formulation of hydrogel that provides for sufficient BMP2 expression to induce *in vivo* bone formation. We have been working on introducing degradation sites within this material that would allow it to be remodeled by osteoclasts similar to normal skeletal bone. We have synthesized the peptide sites and introduced them into the PEG-DA strands and succeeded in getting normal crosslinking of the material. We have then degraded the hydrogel completely in the presence of either a general protease (proteinase K) or a specific proteinase (cathespin K) but not with other selective proteinases. We are currently testing the hydrogel materials in the presence of cells, either a non-specific fibroblasts (skin fibroblasts) or osteoclasts to demonstrate again the selectively degradable nature of the material. We will then seed the hydrogels with BMP2 transduced cells, and further test these for both BMP2 expression as well as in vivo animal testing and selectable degradation. (we will implement the Ad5E1BMP2E3 dsRED vector for these purposes, so that we can track the expression and degradation in vivo.
- We have previously demonstrated similar temporal and rate of bone formation in comparison studies between C57BL/6 (immunocompetent) mice and the NOD/Scid (immune incompetent) mouse model. We have initiated studies in the spine, to obtain fusion. We have preliminary data which demonstrated rapid spine fusion in mice that received either direct injection of the AdBMP2 transduced cells into the paraspinous musculature or implantation of the same cells after encapsulation within the hydrogel material. In this analysis we consider a fusion as the fusion between the newly induced heterotopic bones with the adjacent vertebral bone. We did not place the criteria of fusion as being two or more vertebra in this initial analysis. One reason we did not constrain the analysis to multiply fused vertebra, was because in our model we are not inducing any injury or surgical material, other than a simple injection. Thus we are dependent on launching heterotopic bone formation that can fuse and remodel

with the adjacent skeletal bone. In this analysis, the hydrogel encapsulated cells more consistently formed bone within the spine, than the direct injection. One reason we suspect that the direct injection is effective but less reliable is the ability for the cells to be sequestered at the site in order to launch the reaction. Since the paraspinous musculature is fairly small, maintaining the cells in this tissue is quite difficult without inclusion of the biomaterial. Thus we propose that the increased number of spines that have fused to at least one vertebra is actually greater when the cells are maintained at the specific site. Alternatively, the structure of the hydrogel material is critical to achieving fusion of two or more vertebra and meeting the standard definition of spine fusion. Larger second studies with the rod structured as well as a free formed hydrogel material is underway.

- In these studies we set up a large number of animals for biomechanical testing to develop better criteria for demonstrating true fusion of two or more vertebra. The first groups used for this experimental design for direct injected cells; however, we are currently now testing fusions in animals receiving the hydrogel encapsulated cells. We have developed a biomechanical testing strategy to rapidly demonstrate true spine fusion with the vertebra in our rodent and larger animal models. This is a critical component to characterizing the biomaterial, since on many occasions' microCT or x-ray analysis can appear as a true fusion, however, upon bending the heterotopic bone will break at the fusion site rather than constrain the spine, as a result of poor fusion, and remodeling with the normal skeletal bone.

Reportable Outcomes:

Abstracts: Poster presentation:
Orthopedic Research Society Annual Meeting San Francisco, CA. 2008
7th Annual International Conference on Bone Morphogenetic Proteins. Tahoe CA, 2008

Oral Presentations:
American Society of Bone and Mineral Research Annual Meeting Honolulu HI, 2008.
American Society of Gene Therapy Annual Meeting Boston, MA 2008

Manuscripts:

We have three manuscripts in preparation; however, nothing has been accepted for publication yet. The first outlines the biomechanical testing that we have designed to confirm true fusion in the spine. The second manuscript is demonstrating spine fusion with bone direct injection as well as hydrogel encapsulation. The third manuscript is a comparison study demonstrating the sensitivity of dsRED and its use in tracking cells in vivo.

Conclusions:

We have demonstrated the ability of both the hydrogel encapsulated cells as well as the cells directly injected to induce heterotopic bone formation when implanted in the paraspinous musculature. This heterotopic bone formation can then fuse to the adjacent skeletal bone, even in the absence of injury or decertification. In both cases when cells are directly injected or hydrogel encapsulated the heterotopic bone can form a bridge between two or more vertebra to create spine fusion. Interestingly, although the direct injection appears to be less capable of consistently forming heterotopic bone in the spine model, when bone is formed, it more consistently bridges multiple vertebras leading to fusion. This is most likely due to the structures used in the hydrogel studies, and hence the need for a degradable form of the material, which can be remodeled by newly forming bone. From these initial studies we have then designed a method for demonstrating true fusion of the spine through a biomechanical testing, which will reliable validate and compliment the imaging data. We have demonstrated the ability of the BMP2 transduced cells to induce spine fusion in Nod/SCID mice, through gross anatomical observations as well as biomechanical testing. We have validated that the bone induction mechanism in this model, works similarly in immune competent mice. Finally we have demonstrated the ability to encapsulate the BMP2 transduced cells in a hydrogel material, and still maintain their viability and efficient secretion of BMP2. Further, delivery of the hydrogel encapsulated cells led to targeted bone formation, and vertebral fusion when implanted in a spine setting. These are the first steps in developing a safe and efficacious noninvasive gene therapy for the tissue engineering of bone.

However, for this system to become a reality, we must determine the minimal number of transduced cells required for spine fusion when delivered through this targeted method. Using this information we must next reduce the volume of polymer used for encapsulation to better fit the spatial restrictions found in the spine, and finally we must move towards using the polymer encapsulation system which is photo-polymerized in place rather than preformed structures that must be surgically implanted. We must perform more replicates to identify to develop a time frame for fusion that will provide a ≥ 90% success rate. To aid in these goals we propose to improve both the secretion of BMP2 through re-engineering the BMP2 protein to remove a region known to have strong binding to glycoprotein's on the cell. Previous work in the literature has shown removal of such sites greatly increases the diffusion of secreted proteins. We have currently constructed an adenovirus vector possessing this deleted form of BMP2, and are currently testing it for its ability to launch bone formation similarly to our complete BMP2 vector. Finally we have proposed to add in a regulated promoter system to the expression of BMP2 and demonstrate *in vivo* using a novel imaging methodology sensitive enough to potentially track handfuls of cells to confirm both the localized and regulated expression of BMP2. To this end we have developed vectors possessing the dsRED molecule and are currently further testing the sensitivity of this system to track the cells producing the BMP2.

Completion of this project would significantly advance the current state of gene therapy in this field by eliminating the search for an optimal osteoprogenitor cell and scaffolding. But even more importantly, it would offer a non-invasive alternative to current treatments for degenerative spine disorders. Posterolateral spine fusion, which normally results in 500-1000 cc of blood loss as well as a 5 to 7 day hospital stay and a recovery period of up to a year, could be performed on an outpatient basis with this minimally invasive procedure, without concern over undue morbidity. This technology would benefit a broad age range of patients, and greatly reduce treatment costs as well as loss work time. Our proposed method has the potential to improve the safety of current spine surgery techniques and would offer an alterative to patients who require spine fusion but are not candidates for major surgery.

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2. Zhao KD, Yang C, Zhao C et al. Assessment of noninvasive intervertebral motion measurements in the lumbar spine. *J.Biomechanics* 2005;38:1943-6.